Exploring the potential of plant growth promoting endophytes from *Piper longum* (L.)

Submitted By:

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Synopsis of the proposed work for the award of degree

Doctor of Philosophy

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I. Introduction

Endophytes are bacterial or fungal microorganisms that colonize the internal tissues of plants symbiotically without causing any apparent symptoms of disease (Wilson, 1995). Endophytes are ubiquitous microbes reported from all the plants investigated so far (Dhanya and Padmavathy, 2014). They are taxonomically and ecologically heterogenous groups of organisms comprising mainly of bacteria, fungi and actinomycetes (Sakina *et al*., 2015). These have been reported to impart various beneficial traits to host plants, especially under stress conditions. Moreover, they serve as alternative sources for many bioactive secondary metabolites (alkaloids, phenolics etc.) and phytohormones such as Indole-3-acetic acid (IAA), ethylene-like, cytokinine-like and gibberellins-like compounds (Subbulakshmi *et al*., 2012).

II. Review of literature

Endophytes refers to the microorganisms that occurs within plant tissues, distinct from epiphytes that live on plant surfaces (Bacon *et al*., 2000). Many plant processes have been shaped through association with endophytic fungi. For example, endophytic fungi are suggested to play a major role in structuring plant communities and in shaping processes such as colonization, competition, coexistence and soil nutrient dynamics (Saikkonen *et al.*, 2002). Endophytic fungal diversity is shaped by environmental or habitat condition in which the plant take resistance. Endophytes promote phosphorous solubilization, nitrogen fixation and suppression of stress related ethylene synthesis in plants through the production of 1-aminocyclopropane-1- carboxylate (ACC) deaminase (Hardoin *et al.,* 2008; Rosenblueth and Romero, 2006; Vega *et al.,* 2010). Endophytes also facilitate biocontrol activity by protecting plants against pathogens. They confer plant's defense mechanism through the production of substances like siderophores, antibiotics or by competing with pathogenic organisms for colonization sites and nutrients (Rosenblueth and Romeo, 2006).

Medicinal plants are the most promising source for various natural bioactive products and secondary metabolites producing endophytes which have the ability to target specific proteins coded by essential genes (Kingston, 2001). Many bioactive metabolites are originated from fungal endophytes that have wide potential to produce numerous metabolites with antimicrobial properties (Suryanarayanan *et al*., 2009). Endophytes are also known to produce a large number of secondary metabolites as a source of pharmaceutically important compounds. Since the discovery of taxol from endophytic fungus, *Taxomycesandreane*, researchers have been encouraged to select medicinal plant for endophytic study that have well known therapeutic as well as ethnobotanical history which contain a well characterized set of chemicals (Wani *et al*., 1971).

Endophytic fungi also produce a large number of metabolites as demonstrated by a number of fungal culture studies (Tan and Zou 2001). The metabolites including alkaloids, steroids, terpenoids, isocoumarins, quinones, flavonoids, phenylpropanoids, lignans, peptides, phenolics and volatile organic compounds have raised tremendous interest especially from the possibility of exploiting the fungi as source of pharmaceutically important compounds (Tan and Zou 2001; Gunatilaka 2006; Zhang *et al.*, 2006). Endophytes isolated from the petiole and internodes of *Piper longum* were found to produce indole-3-acetic-acid (IAA), the phytohormone with growth promoting properties and three endophytes were also isolated which were found to produce hydrogen cyanide (Mubashar *et al*.,2018). Piperine, the principal metabolite present in *Piper nigrum* (Kiuchi *et al*., 1988) is mainly responsible for the spiceness of the pepper. It was first discovered by Hans Christian Orsted in 1819 (Orsted, 1820). Piperine is reported to have a wide pharmaceutical properties including antibacterial, antifungal, hepato- protective, antipyretic, anti-inflammatory (Parmar *et al*. 1997; Mittal and Gupta 2000) and anti-tumor effects (Sunila and Kuttan, 2004). But unfortunately very few reports on endophytic fungi concerning piperine production from *Piper longum* is available in the literature. A piperine producing fungus, *Ulocladium* species was isolated from *Piper longum* (Dahiya *et al.,* 1998) but this report do not provide the biology and ecology of the fungus. An endophytic fungus, *Periconia sp*. was isolated from *Piper longum* which

was able to produce piperine and thus the fungus has antimycobacterial activity (Verma *et al*., 2011). A piperine producing fungus *Colectotrichum gloeosporioides* was isolated and characterized from *Piper nigrum*, the close relative of *Piper longum*. This particular fungus has the potential to be exploited for the large scale production of piperine by scaling up the culture conditions (Chithra *et al*., 2013).

Endophytes are also well known for their role in promoting plant growth and development (Palaniyandi *et al*., 2013). Several plant associated bacteria have shown beneficial impact on the overall health of the plant like bacteria residing in the proximity of a plant's root are termed as plant growth promoting rhizobacteria (PGPR) (Calvo *et al*.,2014; Glick, 2014). PGPR are known to produce a class of phytohormones known as auxins (Duca *et al.*, 2014). Indole-3-acetic acid (IAA) is the most abundantly produced auxin by strains of PGPR, however several report suggest that Indole-3-butyric acid (IBA) has also been produced by these strains (Vessey, 2003; Erturk *et al.*, 2008; Liu *et al*., 2013). IAA is a common product of L-tryptophan metabolism produce by several microorganisms including plant growth promoter Rhizobacteria (Lynch, 1985). Bacteria inhabit the rhizosphere and enhance plant growth by any mechanism through production of plant growth regulators (like auxin, gibberellin and ethylene), siderophores, HCN and antibiotics (Arshad *et al*., 1992). Bacteria synthesize auxins in order to perturb host physiological process for their own benefit (Shih-Yung, 2010). IAA produced by fungi can induced lateral root formation and root hair development (Ludwig-muller, 2015). Under invitro condition it has recently been recognized that various endophytic fungi produced GA and IAA. It has recently been established that certain fungi including endophytes harbor them in their growth medium (Pieterse CM *et al.* 2009).

Endophytes also have the ability to reduce host susceptibility to abiotic and biotic stresses including heat, salt and drought stress (Rodriguez *et al.*, 2008). Fungal endophytes provide fitness benefits (Brundrett, 2006) to plants by increasing root and shoot biomass, by increasing yield and by increasing tolerance to abiotic stresses such as heat, salt and drought and biotic stresses such as pathogens and herbivores (Arnold *et al*., 2003 ; Chaw *et al*., 2004). One particular group of fungal endophytes have

the potential to provide habitat specific stress tolerance to plants through a process defined as Habitat Adapted Symbiosis (Rodriguez *et al.,* 2008). Recent studies also indicates that fitness benefits provided by mutualistic fungi contribute to or are responsible for plant adaptation to stress (Stone *et al.,* 2003; Rodriguez *et al*., 2004). Symbiotic fungi can reduce host plant susceptibility to drought, metals disease, heat and herbivory and promote growth and nutrient acquisition. It has also been reported that at least some plants are unable to tolerate habitat impose abiotic and biotic stress in absence of fungal endophytes (Redman *et al*., 2002).

III. Objectives

The present study will be focused on the isolation and characterization of bacterial and fungal endophytes from *Piper longum,* with special emphasis on exploring the role of plant growth promoting endophytes on growth and development of some selected plant species. *P. longum*, belonging to family Piperaceae, is an important medicinal plant of India, also known as Long Pepper (in English) and Pipali (in Hindi). It is a flowering vine cultivated for its fruit, which is usually dried and used for culinary and medicinal purposes. Long pepper has a taste similar to but hotter than its close relative *Piper nigrum*andis a major source of secondary metabolites like piperine, piper longumine, sesamin etc. It is most commonly used to treat chronic bronchitis, asthma, constipation, chronic malaria, paralysis of the tongue etc.

The present study is proposed to be undertaken with the following objectives:

1. Isolation of bacterial and fungal endophytes in different seasons from various parts of *Piper longum* by Culture-dependent approach.
2. Molecular identification of the isolated endophytes by ribosomal RNA sequencing.
3. Screening of the isolated endophytes for their ability to produce plant growth promoting substances and other beneficial traits.
4. Qualitative and quantitative analysis of plant growth promoting substances produced by the endophytes.

5. Effect of the isolated plant growth promoting endophytes on growth parameters of some selected plant species.

IV. Methodology

Endophytes will be isolated from healthy plants of *Piper longum* maintained in Phal Bagh of Dayalbagh Educational Institute, Agra.

1. Surface sterilization and isolation of endophytes

Different partsof *P. longum* plant will be surface sterilized and inoculated on NA (Nutrient Agar) and PDA (Potato Dextrose Agar) medium, for isolation of endophytic bacteria and fungi, respectively. According to the protocol of Verma *et al*., 2011, the proper surface sterilization of plant parts shall be confirmed by two methods: (i) taking imprints of sterilized plant parts, and (ii) spreading 0.1 ml of last rinsed water, on PDA and NA plates. Absence of any microbial growth on these plates after 4-7 days of incubation at 26 ºC to 28 ºC should confirm the effectiveness of the sterilization procedure.

2.Identification of the isolated endophytes by ribosomal RNA sequencing

The bacterial and fungal isolates will be identified by using ribosomal RNA sequencing method or other similar approach. Genomic DNA from the isolates will be extracted and used as template for PCR amplification using specific primers. The amplified DNA will be sequenced and identified using BLAST tool of NCBI.

3. Screening of endophytes for Plant growth promoting properties

In the present study, the plant growth promoting endophytes shall be screened by their ability to produce either one of the phytohormones or any activity that facilitates nutrient acquisition from fixed nitrogen, iron, phosphate, zinc etc. Some of the proposed protocols have been mentioned below.

(a) Indole-3-acetic acid production

Indole -3-acetic acid (IAA) was the first phytohormone to be discovered and plays key role in plant growth and development throughout the life cycle. IAA is generally considered to be the most important native auxin. Some of the key physiological responses include stem and coleoptile growth, leaf development, vascular differentiation and fruit development.

In order to screen for the endophytes producing IAA, a colorimetric assay will be performed following the protocol of Ehmann, 1977. The isolates of fungi will be inoculated in PDA broth and bacteria in NA broth, and incubated at 26 to 28 ºC at 120 rpm for 72 hour in case of bacteria (Patten and Glics, 2008) and for 4 days in case of fungi (Tang and Bonner, 1977). The broth will be centrifuged and the supernatant will be mixed with Salkowski's reagent and kept in dark for 30 minutes. The 'positive control' tube shall contain only broth (without endophytic microorganism) and standard IAA; while the 'negative control' tube shall contain only broth. The presence of IAA will be indicated by the appearance of pinkish color of the supernatant.

For quantitative estimation, IAA positive strains will be again inoculated in YMD (Yeast Malt Dextrose) broth in case of fungi and LB (LuriaBertani) broth in case of bacteria with tryptophan or without tryptophan and incubated at 26 to 28 ºC for 7 days in case of fungi (Anjali *et al.*, 2013) and for 3 days in case of bacteria (Patten and Glics, 2008) at 120 rpm. The broth will be centrifuged and the supernatant will be mixed with ethyl acetate (1:2) and shaken vigorously. After vigorous shaking it will be allowed to stand for 10 min. IAA will be extracted with solvent layer and the ethyl acetate will be allowed to evaporate. The crude IAA collected will be suspended in methanol. For qualitative estimation, the optical density (OD) of the crude extract will be recorded at 530 nm after 30 and 120 min. IAA production should also be compared with and without tryptophan. Quantitative estimation of the crude extract will be done through HPLC technique. To study the effect of IAA pots assay can be performed using a suitable plant to check the rooting ability and other growth parameters by applying different quantity of the IAA extract.

(b) Gibberellin production

Gibberellins (GA) are growth hormones and stimulate cell elongation and cause plants to grow taller. They also stimulate stem growth, regulate transition from juvenile to adult phase, floral initiation, fruit set and parthenocarpy and seed development and seed germination.

Low GA/dwarf cultivars of rice can be used to screen for fungal and bacterial isolates producing GA (Khan *et al*., 2008). To check the production of GA, fungal isolate should be cultured in czapek broth (1% glucose and 1% peptone) for 7 days and bacterial isolate in LB broth for 72 hours at 120 rpm at 26 to 28 ºC and the culture biomass should be separated by centrifugation. 10µl of the supernatant diluted with distilled water should be applied on apices of sterilized seedlings of dwarf rice cultivars at 2-leaf stage. One seedling should be treated with standard GA for the 'positive control' and one with distilled water for the 'negative control'. Seedling should be harvested after 1 week of the supernatant treatment and different growth parameters should be noted. The GA treated seedlings should also be compared with the positive and negative control. The quantification of GA will be carried out using suitable techniques.

(c) Cytokinin production

Another important class of phytohormones are cytokinins that have been reported to regulate cell division in shoots and roots by controlling specific components of the cell cycle, promote lateral bud growth, delay leaf senescence and promote movement of nutrients.

Screening of the endophytic isolates for cytokinin production will be carried out following the protocol of Fletcher *et al*., 1982. Pure culture of each putative endophyte will be cultivated separately in a shaker incubator in culture tubes containing 10ml of LB broth and PDA broth for bacteria and fungi respectively at 26 to 28 ºC at 120 rpm. After an overnight culture, cell free broth will be extracted and mixed with an equal volume of ethyl acetate. Ethyl acetate extract will be collected by vigorous shaking and will be evaporated to obtain dry extract. The dry extract will again be dissolved in 1ml analytical grade ethanol. Finally the ethanol will be evaporated at 50 ºC and the dried extract will be dissolved in

methanol. The crude extract will be applied on cotyledons germinated from sterilized seeds of any suitable crop plant for the particular assay. One cotyledon should be treated with standard cytokinin (BAP) for the 'positive control' and one with distilled for the 'negative control'. The cotyledons should be observed after one week of incubation in the dark. Greening of cotyledons indicates the presence of cytokinin. The cotyledons along with positive and negative control will be incubated under fluorescent light for 3.5 hours at 27 ºC ± 2. The cotyledons will be ground with 80% acetone with motar and pestle after the incubation. The chlorophyll extract will be collected and centrifuged at 4000 rpm for 10 min. The derived supernatant will be analyzed for total amount of chlorophyll using spectrophotometer (663nm and 645nm).

(d) Other plant growth promoting/beneficial traits

The isolated endophytes shall be screened for their ability for nutrient acquisition under fixed/unavailable forms of nutrients like phosphate, potassium, zinc, iron etc. For phosphate solubilization, the protocol of Jasim *et al*., 2013 using Pikovskaya medium and bromophenol blue as indicator will be followed. The ability of endophytic isolates to produce ammonia shall be assessed using Nesseler's reagent in peptone liquid media (Singh *et al*., 2014) where the intensity of colour change indicates endophytic capacity for ammonia production. The property of the isolated endophytes will also be checked for the production of potassium and zinc oxide using Aleksandorf medium and modified Pikovskaya medium, respectively. Qualitative productions of siderophores by bacterial cultures will be detected on the Chrome-azuorol S medium (CAS medium) as described by Schwyn and Neilands. Each endophytic bacterial isolate will be inoculated on the surface of CAS agar plates and incubated at 28±2 ºC for 72 h. The plates will be observed for colour change i.e. orange to yellow halo zone around the bacterial colonies.

In addition, the isolated endophytes shall also be screened for some enzymes having industrial importance like amylase, cellulose, pectinase and xylanase. The screening for production of these enzymes shall be done by growing the endophytic isolates on media supplemented with 1% of soluble starch, cellulose or carboxy-methylcellulose (CMC), gelatin, pectin and xylan, respectively. The

appearance of clear zone will be measured after adding reagents (iodine, acidic mercuric chloride, hexadecyl trimethyl ammonium bromide and absolute ethyl alcohol to detect the amylolytic, cellulolytic, proteolytic, pectinolytic, xylanolytic activities respectively) and used as indicator for extracellular enzymatic activities (Fouda *et al*., 2015).

4.To study the effect of the isolated endophytes on plant growth in some selected plant species

Promising strains of endophytes with plant growth promoting traits will be inoculated in some economically important plant species. Thereafter, their physiological and growth parameters like seed germination, rooting and shooting, stem elongation, leaf expansion, rate of photosynthesis and fresh and dry weight of different plant parts will be analyzed.

V. Importance of the study

In the present scenario of increasing human population and limited resources, it is necessary to increase agricultural productivity in a sustainable manner. This is because large scale use of chemical fertilizers has led to soil deterioration, water pollution, and negative impact on the entire ecosystem. One of the ways to achieve sustainable agriculture is through the use of plant growth promoting endophytes, which could be used as biofertilizers or biocontrol agents. Other advantages of using plant growth promoting endophytes include cost-effectiveness, easy to access and simple mode of application.

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